ELSEVIER

Urologic Oncology: Seminars and Original Investigations 37 (2019) 530.e9–530.e18

UROLOGIC ONCOLOGY

Laboratory-Bladder cancer

Prognostic value and association with epithelial-mesenchymal transition and molecular subtypes of the proteoglycan biglycan in advanced bladder cancer

Gerald B. Schulz, M.D.[a,*], Tobias Grimm, M.D.[a], Christine Sers, Ph.D.[c], Pamela Riemer, Ph.D.[c], Manal Elmasry, MSc[b], Thomas Kirchner, M.D.[b], Christian G. Stief, M.D., Ph.D.[a], Alexander Karl, M.D.[d], David Horst, M.D.[c,*]

[a] *Department of Urology, Ludwig-Maximilians-University, Munich, Germany*
[b] *Department of Pathology, Ludwig-Maximilians-University, Munich, Germany*
[c] *Department of Pathology, Charité Universitätsmedizin, Berlin, Germany*
[d] *Department of Urology, Barmherzige Brüder, Munich, Germany*

Received 15 August 2018; received in revised form 14 April 2019; accepted 13 May 2019

Abstract

Objective: Dysregulation of the extracellular matrix molecule biglycan (BGN) predicts poor survival in several cancer entities. Our study investigated the prognostic impact of BGN in bladder cancer (BC) in 2 independent cohorts and assessed its role in epithelial-mesenchymal transition (EMT) and association with molecular BC subtypes.

Methods: BGN protein expression was correlated with the oncological outcome of 162 patients with BC undergoing radical cystectomy (RC) in a single center and furthermore on gene expression level in the TCGA database. Cut-off values for BGN protein and RNA expression were tested with receiver operating characteristic (ROC) curves. BGN gene expression was correlated with established EMT and BC gene signatures in the TCGA database using gene set enrichment analysis (GSEA). Key EMT and basal/luminal molecular BC subtype markers were correlated with BGN expression and data were shown in a heat map.

Results: BGN upregulation in BC cells on the protein level predicted poor oncological survival in the institutional cohort for both univariate ($P = 0.007$) and multivariate ($P = 0.040$) analyses. BGN expression was not associated with other clinicopathological parameters. The prognostic value of BGN was validated on the mRNA level in the BC TCGA database ($P = 0.002$). Both EMT and BC core gene signatures ($P < 0.001$) correlated with BGN expression in GSEA. BGN gene expression was associated with key indicators of EMT. BGN was associated positively with the molecular basal BC subtype and negatively with the BC luminal subtype.

Conclusion: BGN is an independent prognosticator for poor survival in BC patients. BGN is associated with the basal molecular BC subtype. EMT might be a key player for BGN driven oncogenesis, as BGN expression correlates with EMT gene signatures.

Keywords: Biglycan; Urinary bladder neoplasms; Cystectomy; Epithelial-mesenchymal transition; Proteoglycans; Biomarkers; Molecular typing

Abbreviations: BC, bladder cancer; NMIBC, nonmuscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; RC, radical cystectomy; EMT, epithelial-mesenchymal transition; BGN, biglycan; GSEA, gene set enrichment analysis; TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; CSS, cancer-specific survival; OS, overall survival; ECM, extracellular matrix

1. Introduction

For advanced bladder cancer (BC), prognosis is still poor, with a 10-year cancer-specific survival (CSS) ranging under 17% in lymphatic spread tumors [1]. Although immune-checkpoint inhibitors have recently revolutionized

Funding: Supported by a grant of the Rudolf Bartling Stiftung (to DH).

*Corresponding authors. Tel.: +49 89 4400 72971; fax: +49 89 4400 78890 (G.B.S.); Tel.: +49 30 450 536 001 (D.H.).

E-mail addresses: gerald.schulz@med.uni-muenchen.de (G.B. Schulz), David.horst@charite.de (D. Horst).

https://doi.org/10.1016/j.urolonc.2019.05.011
1078-1439/

treatment options for metastasized urothelial cell carcinoma, the overall response rate ranges around 20%–50% [2]. In addition to novel therapeutic targets, there is a need of prognostic and predictive markers as it is still unclear, which patients will have the greatest benefit from adjuvant or neoadjuvant therapy [3,4]. Furthermore, a deeper understanding of molecular BC tumor biology is necessary in addition to ongoing clinical trials.

The small-leucine rich proteoglycan biglycan (BGN) has been associated with advanced tumor stages and adverse oncological outcome in several tumor entities, including melanoma, pancreatic, endometrial, gastric, colorectal, and prostate cancer, as well as esophageal adenocarcinoma [5–12]. For BC, the situation is rather unclear. A study showed elevated BGN levels in the blood of BC patients and increased BGN expression in BC tissue compared to healthy controls [13]. However, another investigation correlated BGN up-regulation on RNA level with improved survival in a cohort of BC patients [14].

Initially, the function of BGN has been primarily seen in maintaining the structural integrity of the extracellular matrix (ECM). However, within the last years, BGN has been established as a signaling molecule mediating different steps of carcinogenesis [15].

The regulation of epithelial-mesenchymal transition (EMT), one of the hallmarks of cancer, seems to be regulated partially by BGN [16,17]. During EMT, tumor cells detach from the main tumor mass by attaining mesenchymal characteristics. Within this process, which is a precondition for both tumor cell invasion and metastasis, cancer cells remodel the ECM, of which BGN is part of. On the molecular level, EMT is characterized by the increased expression of several transcription markers like SNAI1, ZEB1 and TWIST1, ECM components (FN1) and mesenchymal markers (VIM) together with the down-regulation of epithelial markers including CDH1 (E-Cadherin). Importantly, EMT has been shown to be relevant also in BC [18,19]. EMT has been associated with tumor resistance mechanisms and therefore underlines potential therapeutic implications [20].

The molecular subtyping of BC has revolutionized the understanding of BC [21]. Although different classification systems have been proposed, the inclusion of a basal and luminal subtype seems to be a basic consensus [22]. Importantly, molecular subtyping might have direct clinical implication, as a previous study demonstrated improved efficacy of neoadjuvant chemotherapy in basal subtype BC patients [23].

In this study, we investigated the prognostic role of BGN in advanced BC in an institutional cohort on the protein level and in addition in an external transcriptome data set from the TCGA database. The association of BGN and EMT was assessed using different gene expression signatures and key EMT drivers. Furthermore, BGN gene expression was correlated with luminal and basal molecular BC subtypes.

2. Methods

2.1. Clinical cohort

One hundred sixty-two patients who underwent RC between 2004 and 2014 in our institution because of muscle-invasive bladder cancer (MIBC) were included. Exclusion criteria were nonmuscle-invasive BC (NMIBC), lack of follow-up data, absence of written consent, and inadequate quality of pathologic tissue. For further stratification, we also excluded all patients with metastatic BC (M1), positive surgical margins (R1/R2), non high-grade bladder cancer, and neoadjuvant chemotherapy. All patients signed a written consent form for clinical follow-up. Specimen were retrieved from the archives of the institute of pathology and all data were anonymized. Need of consent was waived by the institutional ethic committee of the Medical Faculty of the Ludwig-Maximilians-University Munich (LMU).

Tissue-microarrays (TMA) with 1 mm cores from the tumor center and the tumor edge were assembled in triplicates. Hematoxylin-eosin-stained tissue samples were used as templates. Samples of normal urothelium were included. Follow-up was done by questionnaires sent by mail in regular predefined intervals as well as telephone interviews. All histopathological samples underwent an expert pathological review regarding grading and staging parameters. For disease-specific survival, BC-associated death was defined as clinical endpoint.

2.2. Immunohistochemistry

Five micrometer TMA sections were cut, deparaffinized, and stained with a BGN antibody with UltraView Universal DAB detection kits on a Ventana Benchmark XT autostainer (Ventana medical systems). A polyclonal rabbit anti-BGN antibody (HPA003157, Sigma-Aldrich) was used in a 1:300 dilution. Quantitative evaluation of protein expression was done by ImageJ. Cut-off levels for protein expression were determined by using receiver operating characteristics (ROC) curves, and the area under the curve (AUC) is depicted in the respective figures. Importantly, the quantitative assessment of BGN protein expression with ImageJ included only BC cells, while all stromal, ECM, and interstitial tissues were excluded.

2.3. BC TCGA, gene set enrichment analysis (GSEA), heat maps

In order to validate the role of BGN in BC, we used gene expression data (RNA-Seq) of The Cancer Genome Atlas (TCGA) (https://gdc.cancer.gov) and corresponding clinical follow-up [24]. BGN cut-off levels for dichotomization were determined by ROC curve analysis. In order to correlate BGN expression with oncogenetic signatures, we performed a gene set enrichment analysis (GSEA). Therefore, we calculated a ranked list of the correlation of BGN

expression with 20,531 genes. Hallmark and curated gene sets with 1,000 permutations using the Molecular Signatures Database v5.0 (Broad Institute) were then correlated to this ranked list [25]. BGN expression with EMT and BC gene signatures and the respective enrichment scores were correlated. Additionally, several EMT regulators and luminal/basal molecular BC subtype indicators were correlated with BGN expression on the mRNA expression level (Spearman's rank correlation coefficient) and heat maps were generated using GENE-E software (Broad Institute).

2.4. Statistical analysis

The impact of BGN on oncological prognosis based on the institutional cohort was tested according to the Kaplan−Meier method and a log-rank test for CSS and overall survival (OS). Additionally, hazard ratios (HR) for the univariate survival analyses were assessed by Cox regression and results are depicted in the respective figures. Together with the Kaplan−Meier plots, the number of events (disease-specific death for CSS; any death for OS) over the number of patients per subgroup is demonstrated as ratios for each subgroup.

Different clinicopathological parameters (age, gender, T-stage, [adjuvant] chemotherapy, [adjuvant] radiotherapy, lymphovascular invasion [LVI], and lymphatic spread) were tested using univariate Cox regression. The impact of BGN protein expression on CSS and OS was tested by multivariate Cox regression analysis. Parameters included for multivariate analyses were tumor stage (pT3/4 vs. pT2), lymphatic spread (N1/NX vs. N0), LVI [26], gender [27,28], age, (adjuvant) radiotherapy, and (adjuvant) chemotherapy.

Key clinical along with histopathological parameters were tested for statistical association with BGN protein expression with Pearson's chi-squared test. For external validation purposes, a survival analysis with CSS as primary endpoint was done for BGN on RNA level (TCGA) and results were demonstrated as plots of the Kaplan−Meier estimator.

Correlations of BGN expression with gene signatures (GSEA) were performed with Pearson's chi-squared test. Statistical tests were performed with IBM SPSS 25. P values <0.05 were considered statistically significant.

3. Results

3.1. BGN expression is strongly associated with poor prognosis

We first analyzed BGN protein expression in normal urothelial tissue and urothelial bladder cancer. BGN was expressed in the cytoplasm of the upper luminal urothelial layer in normal urothelial tissue, but not in basal cell layers. Importantly, BGN also stained in the ECM and at least in some mesenchymal cells (Fig. 1A). Examples of immunohistochemical staining of urothelial BC of BGN high and low subgroups located BGN in the cytoplasmic compartment. BGN seemed to be evenly spread in the cytoplasm of urothelial BC cells, in contrast to normal urothelial tissue, where BGN was expressed only in the luminal part of luminal urothelial cells (Fig. 1B).

Next, we tested for correlations of BGN expression and patient survival. In our collection, the mean follow-up was 3.62 years, and 34.6% and 20.4% underwent adjuvant chemotherapy and/or radiotherapy, respectively. BC-associated death occurred in 89 cases (54.9%). We defined BGN high and low subgroups based on protein expression. The cut-off level for dichotomization of the BC patient cohort into BGN protein expression high and low subgroups is shown together with the respective ROC curve. With this cutoff, $n = 27$ (16.7%) of patient cases were BGN high and $n = 135$ (83.3%) were BGN low (Fig. 1C/Table 1). Survival analysis demonstrated poor prognosis for the BGN high subgroup regarding CSS ($P = 0.007$) and a tendency toward poor outcome for OS ($P = 0.054$) in Kaplan−Meier plots (Fig. 1D and E).

We then analyzed associations with other core clinical parameters. Detailed clinicopathological patient characteristics are shown in Table 1. Clinical (age: >median vs. ≤median, gender: male vs. female, [adjuvant] radiotherapy, and [adjuvant] chemotherapy) and histopathological (T-stage, lymphatic spread/N-category, LVI) characteristics were not associated with BGN protein expression (Table 1).

3.2. BGN expression independently predicts adverse CSS in BC

To further learn about the prognostic power of BGN, we performed univariate and multivariate survival analyses. In univariate Cox analyses, (adjuvant) radiotherapy (HR: 3.345, 95% confidence interval [CI]: 1.951−5.736, $P < 0.001$) and lymphatic spread (HR: 1.814, CI: 1.084−3.036, $P = 0.023$) were significantly associated with CSS in addition to BGN (HR: 6.955, CI: 1.227−4.020, $P = 0.008$; Table 2A). In multivariate Cox regression analysis together with key clinicopathological parameters, BGN proved to function as an independent prognostic predictor for worse CSS (HR: 1.923, CI: 1.032−3.585, $P = 0.040$). Lymphatic spread/N-category pN0 vs. pN1/NX, (HR: 0.503, CI: 0.291−0.869, $P = 0.014$) and (adjuvant) radiotherapy (HR: 3.567, CI: 2.010−6.332; $P < 0.001$) were significantly associated with adverse outcome as well, underlining the validity of this institutional cohort (Table 2B). However, in multivariate analysis for OS, BGN was not a significant prognostic predictor (Table 2C).

These findings suggested that BGN might indicate BC patients with poor prognosis, especially in regards to cancer-specific outcome, while its prognostic power appeared to be independent of other core clinical variables.

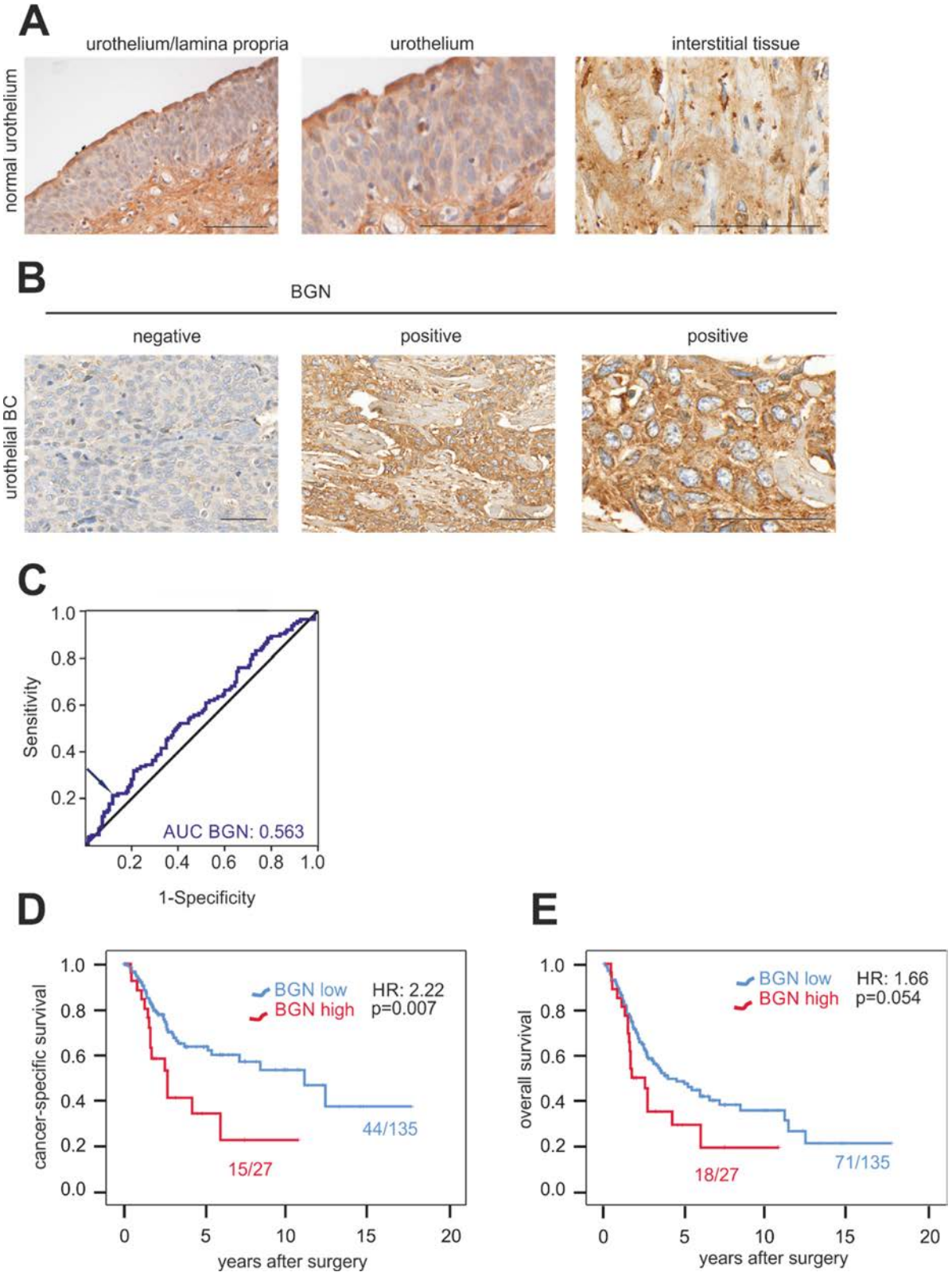

Fig. 1. BGN expression predicts poor survival in urothelial BC. (A) BGN staining in normal urothelial tissue. Urothelial layer of the urinary bladder and magnified sections of the urothelium and interstitial tissue of the lamina propria. Scale bar, 100 μm. (B) Immunohistochemical assessment of high and low BGN protein expression in radical cystectomy samples of patients with muscle-invasive urothelial carcinoma. Magnification of BGN positive urothelial carcinoma in order to assess intracellular BGN distribution. Scale bar, 100 μm. (C) The optimal cut-off level for quantitatively assessed BGN expression in urothelial BC was determined by receiver operating characteristics (ROC) curve for BGN protein expression high/low subcohorts. Area under the curve (AUC) (D, E) Kaplan–Meier plots for the different BGN expression categories for cancer-specific survival (CSS) (D) and overall survival (OS) (E). Hazard ratios and *P* values of the log-rank tests are indicated in the diagrams. The number of events (cancer-specific death for CSS and all causes of death for OS) over the number of patients per group are indicated as ratios over the curves.

Table 1
Patient cohort characteristics and association of BGN protein expression with clinical and histopathological parameters. BGN expression was associated with clinical and histopathological subgroups. Pearson's chi-squared test.

		BGN		
Characteristics	Total	Low	High	*P*
All patients	162	135	27	
Age (y, median 68)				
>68	80 (49.4)	67 (83.3)	13 (16.3)	0.888
≤68	82 (50.6)	68 (82.9)	14 (17.1)	
Gender				
Male	116 (71.6)	95 (81.9)	21 (18.1)	0.436
Female	46 (28.4)	40 (87.0)	6 (13.0)	
T-stage (UICC)				
T2	41 (25.3)	36 (87.8)	5 (12.2)	0.356
T3	98 (60.5)	82 (83.7)	16 (16.3)	
T4	23 (14.2)	17 (73.9)	6 (26.1)	
N-category (UICC)				
N0	90 (55.6)	79 (87.8)	11 (12.2)	0.106
N1	61 (37.7)	46 (75.4)	15 (24.6)	
NX	11 (6.8)	10 (90.9)	1 (9.1)	
(Adjuvant) chemotherapy				
Yes	56 (34.6)	46 (82.1)	10 (17.9)	0.768
No	106 (65.4)	89 (84.0)	17 (16.0)	
(Adjuvant) radiotherapy				
Yes	33 (20.4)	27 (81.8)	6 (18.2)	0.794
No	129 (79.6)	108 (83.7)	21 (16.3)	
Lymphovascular invasion (LVI)				
LVI	49 (30.2)	38 (77.6)	11 (22.4)	0.193
LVI0	113 (69.8)	97 (85.8)	16 (14.2)	
Histological subtype				
Urothelial BC	149 (92.0)	124 (83.2)	25 (16.8)	0.811
Squamous BC	11 (6.8)	9 (81.8)	2 (18.2)	
Other BC	2 (1.2)	2 (1.5)	0 (0.0)	
UICC stage				
2	22 (13.6)	20 (90.9)	2 (9.1)	0.526
3	139 (85.8)	114 (82.0)	25 (18.0)	
4	1 (0.6)	1 (0.7)	0 (0.0)	

3.3. BGN predicts poor oncological prognosis in the TCGA database and is correlated with BC-specific gene signatures in GSEA

The external BC TCGA cohort was used for further analysis of BGN biomarker potential on the gene expression level. An optimal cut-off score of 18,650 normalized mRNA reads for BGN gene expression was determined by ROC curve analysis. With this cutoff, $n = 42$ (13.5%) of patient cases were BGN high, and $n = 269$ (86.5%) were BGN low (Fig. 2A). BGN overexpression predicted adverse CSS ($P = 0.002$; Fig. 2B). Furthermore, using GSEA, we found that 2 BC-specific gene signatures, Lindgren BC high recurrence ($P < 0.001$) and Lindgren BC cluster 2B ($P < 0.001$), strongly enriched with increasing with BGN expression [29] (Fig. 2C). These findings further strengthened the prognostic impact of BGN in BC through validation in an independent cohort on the gene expression level and also suggested a possible association with intrinsic characteristics of BC cells.

3.4. BGN correlates with EMT and the molecular basal subtype in BC

Next, we investigated the role of BGN in regards to malignant traits of tumor cells and BC subtypes. Interestingly, we found that BGN gene expression was strongly associated with EMT signatures, EMT key indicators, and BC subtypes on the gene expression level. The ranked gene list regarding BGN expression correlated with the hallmark EMT gene signature ($P < 0.001$) as well as the Taube's EMT core signature [30] ($P < 0.001$) in GSEA analysis (Fig. 3A). Additionally, BGN overexpression significantly correlated with EMT mediators: ZEB1 ($r = 0.704$, $P < 0.001$), ZEB2 ($r = 0.732$, $P < 0.001$), TWIST2 ($r = 0.756$, $P < 0.001$), VIM ($r = 0.819$, $P < 0.001$), SNAI1 ($r = 0.681$, $P < 0.001$), TWIST1 ($r = 0.743$, $P < 0.001$), FN1 ($r = 0.770$, $P < 0.001$), and SNAI2 ($r = 0.236$, $P < 0.001$). The epithelial marker E-cadherin negatively correlated with BGN overexpression ($r = -0.208$, $P < 0.001$). Moreover, the molecular BC subtype of luminal markers negatively correlated with BGN expression: FGFR3 ($e = -0.310$, $P < 0.001$), GATA3 ($r = -0.273$; $P = <0.001$), KRT20 ($r = -0.203$; $P < 0.001$), FOXA1 ($r = -0.478$; $P < 0.001$), CD24 ($r = -0.104$; $P < 0.001$). On the contrary, molecular BC subtype basal markers were positively associated with BGN expression: KRT14 ($r = 0.220$, $P < 0.001$), KRT6B ($r = 0.104$; $P = 0.036$). These results are shown color coded in a heat map (Fig. 3B). In conclusion, 2 different transcriptome-based analyses in the TCGA dataset suggested an association of BGN, EMT, and the molecular basal BC subtype.

4. Discussion

Here, we demonstrate that high levels of BGN protein expression predict poor CSS in patients with MIBC undergoing radical cystectomy. Furthermore, the prognostic power of BGN was independent of other core clinical variables, and we also validated these findings on the mRNA level in a large independent BC cohort from TCGA. These results are in agreement with several other studies in different cancer entities including gastric, colorectal, and prostate cancer, in which an increased BGN level has been established as a molecular marker for poor clinical outcome [5−11]. Interestingly however, studies investigating BGN in BC showed contradicting results. Appuni et al. compared BGN serum enzyme activity between BC patients and healthy controls along with BGN expression in BC tissue compared to adjacent nonmalignant tissue [13]. BGN was overexpressed in the serum of BC patients and furthermore in BC tissue on protein and mRNA level compared to the control groups, suggesting that BGN is relevant in BC. On the other side, a previous study on 76 patients with NMIBC and MIBC suggested that up-regulation of BGN might be associated with a better prognosis. Since these data were based on mRNA analysis, and we here demonstrate that in the TCGA data set BGN is strongly linked to poor outcome

Table 2
BGN is an independent predictor for oncological outcome in the institutional cohort. (A) In an univariate analysis, several key parameters known for their prognostic relevance in BC were tested for their impact on cancer-specific survival. (B, C) Multivariate Cox analysis for BGN as binary classifier including important clinicopathological parameters like T-stage, N-category, lymphovascular invasion (LVI), (adjuvant) chemo- or radiotherapy, gender, and age. Cox regression analysis was performed for cancer-specific survival (B) and overall survival (C). Multivariate Cox regression analysis.

A

	Univariate analysis Cancer-specific survival (CSS)		
Variables	HR	(95% confidence interval)	*P*
Age (≥median vs. <median)	1.22	(0.728–2.034)	0.454
Gender (male vs. female)	1.093	(0.824–1.449)	0.538
T-stage (T3/4 vs. T2)	1.863	(0.963–3.603)	0.064
(Adjuvant) chemotherapy	1.625	(0.971–2.718)	0.065
(Adjuvant) radiotherapy	3.345	(1.951–5.736)	<0.001
Lymphovascular invasion (LVI)	1.139	(0.645–2.010)	0.654
N-category (pN1/NX vs. pN0)	1.814	(1.084–3.036)	0.023
BGN (high vs. low)	6.955	(1.227–4.020)	0.008

B

	Multivariate analysis Cancer-specific survival (CSS)		
Variables	HR	(95% confidence interval)	*P*
Age (≥median vs. <median)	1.269	(0.749–2.151)	0.377
Gender (male vs. female)	1.559	(0.867–2.803)	0.138
T-stage (T3/4 vs. T2)	1.401	(0.702–2.795)	0.339
N-category (pN0 vs. pN1/NX)	0.503	(0.291–0.869)	0.014
Lymphovascular invasion (LVI1 vs. LVI0))	0.788	(0.429–1.477)	0.442
(Adjuvant) radiotherapy	3.567	(2.010–6.332)	<0.001
(Adjuvant) chemotherapy	1.027	(0.590–1.790)	0.924
BGN (high vs. low)	1.923	(1.032–3.585)	0.040

C

	Multivariate analysis Overall survival (OS)		
Variables	HR	(95% confidence interval)	*P*
Age (≥median vs. <median)	1.467	(0.953–2.259)	0.082
Gender (male vs. female)	1.509	(0.944–2.414)	0.086
T-stage (T3/4 vs. T2)	1.438	(0.838–2.470)	0.188
N-category (pN0 vs. pN1/NX)	0.558	(0.358–0.872)	0.010
Lymphovascular invasion (LVI1 vs. LVI0)	1.188	(0.748–1.886)	0.465
(Adjuvant) radiotherapy	2.318	(1.412–3.807)	0.001
(Adjuvant) chemotherapy	0.770	(0.483–1.229)	0.273
BGN (high vs. low)	1.358	(0.795–2.320)	0.263

on the mRNA level, we suggest that these contradicting results may be caused by the relatively few cases that were investigated in the previous study [14]. However, since here we found no significant association of BGN expression and OS, we also suggest that the predictive power of BGN may not be prone to competing age-related risks. Considering that our analysis of BGN on the protein level included cancer cells only but the mRNA profiles from TCGA likely also included tumor surrounding stromal cells, we propose that the prognostic power of BGN does not depend on nor is it significantly affected by stromal expression.

Next, we investigated the association of BGN with important clinicopathological parameters. In contrast to investigations in colorectal, gastric and prostate cancer, BGN expression was not associated with advanced tumor stage in our analysis [7–9]. Importantly, our study cohort was highly stratified, excluding metastasized BC (M1), positive surgical margin, patients undergoing neoadjuvant chemotherapy and NMIBC. Therefore, a possible explanation might be a rather homogenous cohort excluding key variables of tumor progression. Furthermore, BGN might play a divergent biological role independent of grading and staging variables in BC.

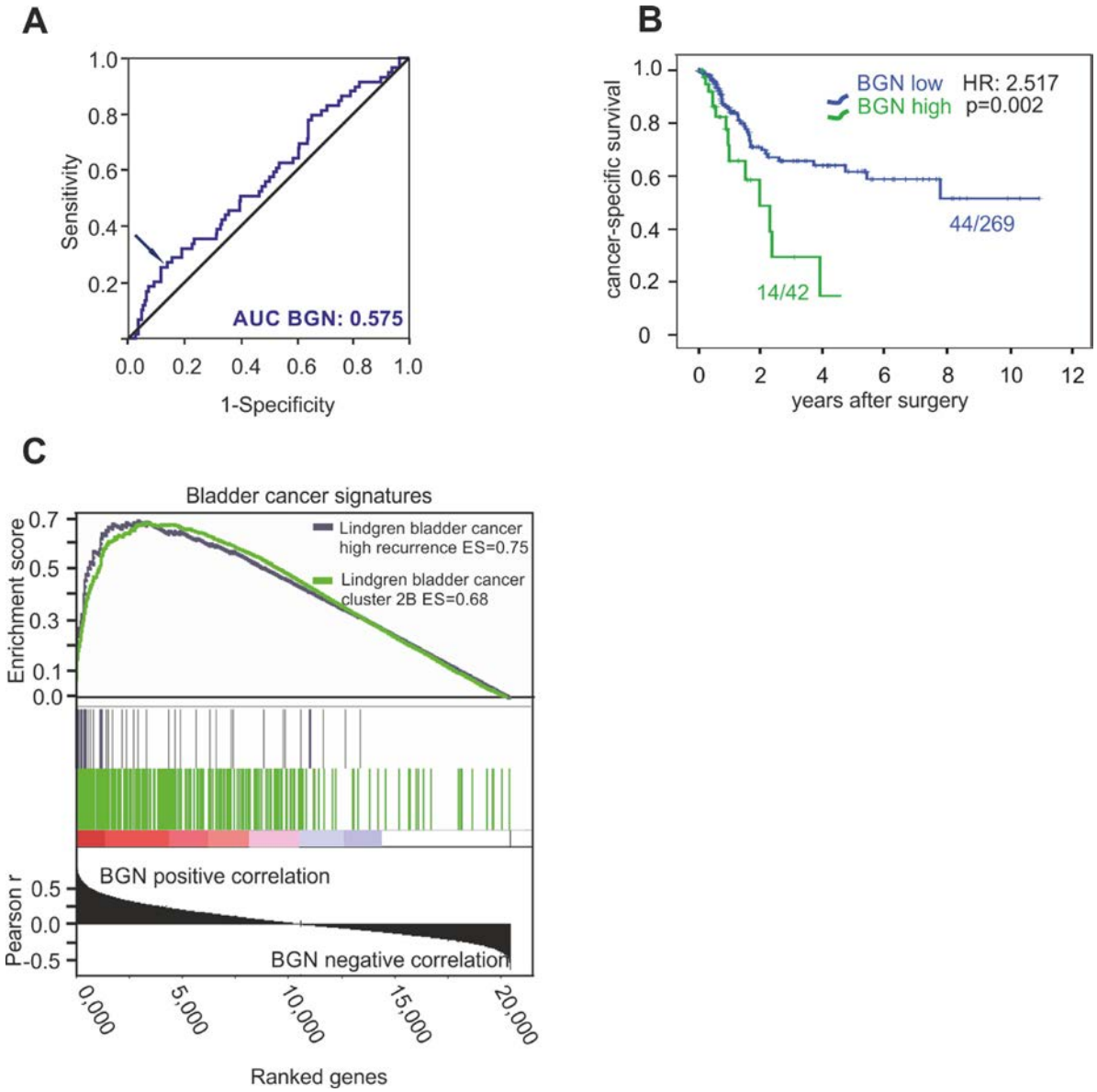

Fig. 2. The correlation of BGN with adverse oncological prognosis is validated in the external TCGA database. (A) Receiver operating characteristics (ROC) curve for BGN gene expression high/low subcohorts. Area under the curve (AUC). (B) Classification of cases by dichotomization with this cutoff demonstrated an association of BGN high expression subgroup with poor disease-specific survival. Results are demonstrated as Kaplan–Meier plots. Number of events (disease-specific death) over the number of patients per group are indicated as ratios over the curves. (C) Gene set enrichment analysis (GSEA) for genes ranked by correlation (Pearson *r*) to BGN expression for 2 core BC gene signatures by Lindgren et al. in gene expression data sets. $P < 0.001$. ES = enrichment score.

We then correlated BGN with BC gene signatures on the gene expression level. GSEA revealed a significant correlation of BGN up-regulation with different BC gene signatures, including "Lindgren BC high recurrence" and "Lindgren BC cluster 2B." These gene sets are based on an expression analysis of 75 NMIBC samples, which resulted into the finding of 4 different gene signatures as well as a cluster of 49 genes associated with a short recurrence-free follow-up [29]. Interestingly, BC cluster 2B was associated with genes regulating both ECM remodeling and immune response in a gene ontology analysis. These data strengthen our hypothesis, as BGN is part of the ECM. On the other hand, this makes BGN a valuable marker to be validated in the setting of immune checkpoint inhibitors. However, it remains to be determined if these data can be directly extrapolated to MIBC, since the BC-specific gene sets were derived from NMIBC.

Other proposed mechanisms of BGN in the oncologic setting include inhibition of the tumor suppressor PTEN [9], induction of the antiapoptotic MAPK pathway [31] and facilitation of cancer cell invasion through activation of the focal adhesion kinase (FAK) pathway [8]. BGN has also been shown to induce tumor angiogenesis by interaction with its receptor TLR2/TLR4 and up-regulation of VEGF

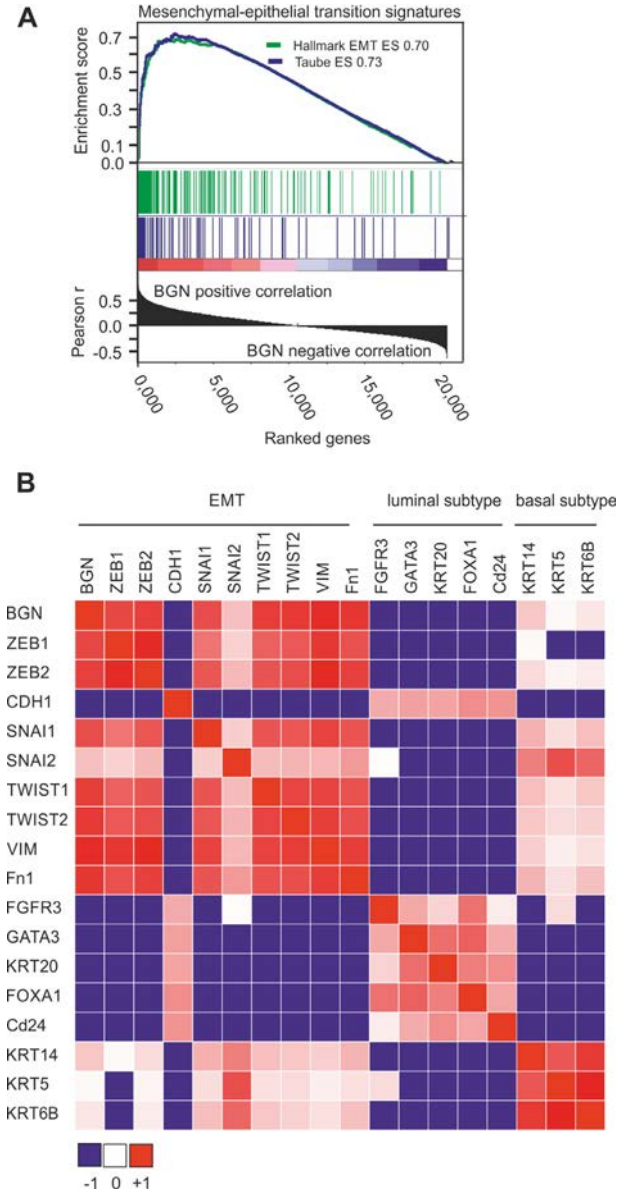

Fig. 3. BGN expression is linked to EMT and bladder cancer basal subtype gene expression in the BC TCGA dataset. (A). Genes ranked by correlation (Pearson *r*) to BGN expression based on the BC TCGA database demonstrated enrichment of the Taube et al. EMT gene set and Hallmark EMT gene set in the gene set enrichment analysis (GSEA). $P < 0.001$. (B) In a heat map graphic positive and negative correlation of different EMT (ZEB1, ZEB2, CDH1, SAIL1, SNAIL2, TWIST1, TWIST2, VIM, FN1), BC luminal subtype (FGFR3, GATA3, KRT20, FOXA1, CD24) and BC basal subtype (KRT14, KRT5, KRT6B) markers with BGN expression in TCGA are demonstrated. Spearman *r* correlation is demonstrated color-coded from −1 (blue) to +1 (red). ES = enrichment score.

[32,33]. This is remarkable, as both the blockage of the receptor together with angiogenesis inhibitors might be interesting therapeutic approaches in BGN overexpressing BC.

We also investigated the correlation of BGN with EMT. EMT core signatures, among them the "hallmark EMT" and the "Taube EMT" gene set, were significantly associated with BGN overexpression in GSEA based on the BC TCGA dataset [30]. Interestingly, EMT and BGN have been linked through the TGFβ pathway, although the exact interplay needs to be clarified in future studies [17]. The tie between BGN and EMT was strengthened by the single-gene correlation of BGN overexpression with established key markers of EMT in a heat map analysis. Importantly, several markers of EMT (ZEB1, ZEB2, SNAI1, SNAI2, TWIST1, TWIST2, FN1, and VIM) showed a positive correlation with BGN expression, whereas the epithelial marker CDH1 (E-Cadherin) showed a significantly negative correlation. Taken together, 2 independent approaches on mRNA level in the TCGA database suggest a link between BGN and EMT. However, it remains to be determined if BGN also functionally contributes to the malignant traits of an EMT phenotype in bladder cancer cells, keeping in mind that stromal expression of BGN also is observed and likely contributes to BGN genes expression levels in BC tissue samples.

EMT itself has been established as a crucial oncogenic process in BC pathogenesis. Up-regulation of E-cadherin (CDH1) has been shown to independently predict better PFS in NMIBC, whereas deletion of E-cadherin was associated with poor prognosis and progressive disease in BC [19,34]. Next to E-Cadherin, the transcription factors TWIST, Snail and ZEB1 were demonstrated to predict recurrence-free survival or advanced stage in BC [35,36]. Notably, in vivo inhibition of the EMT inducing transcription factor Snail has been shown to decrease metastasis [37]. Therefore, the role of EMT in BC might go beyond the implementation of predictive markers, but also might have implications for future BC therapies.

In order to further integrate the role of BGN in our current understanding of BC pathogenesis, we correlated BGN expression with BC molecular subtypes. BGN was positively associated with basal BC subtype markers, and inversely associated with luminal molecular subtype BC markers. Interestingly, the basal subtype was associated with high expression of EMT markers in a previous study [21]. These results are relevant, as molecular subtyping of BC seems to become increasingly relevant for clinical practice [23,38]. Intriguingly, in the histopathological analyses, the luminal distribution of BGN in the normal urothelium was lost in BGN overexpressing BC, where BGN is distributed throughout the cytoplasm. Nevertheless, the exact association of EMT with BC molecular subtypes is still not fully understood and requires further study.

We suggest that BGN is a novel prognostic marker for poor survival in BC and propose its link to EMT as a possible mechanism. However, although a multitude of clinical, laboratory, mRNA, protein along with genomic-based predictors have been evaluated for BC, only very few have reached clinical practice yet [3]. Currently, we are only at the beginning of understanding the role of BGN in ECM remodeling, molecular BC subtypes, tumorangiogenesis as well as EMT in BC. However, there are increasing data on

the drugability of these processes, which will be valuable targets for future research.

5. Conclusion

We link small leucine-rich proteoglycan BGN expression on protein and transcriptome levels in 2 independent cohorts with poor oncological prognosis in BC patients. BGN overexpressing BC is associated with EMT and the basal BC subtype. As EMT is a key pathomechanism for cancer progression and metastasis, BGN might help at identifying patients with the need for more aggressive therapeutic approaches and a closer follow-up.

Conflict of Interest

None.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urolonc.2019.05.011.

References

[1] Hautmann RE, de Petriconi RC, Pfeiffer C, Volkmer BG. Radical cystectomy for urothelial carcinoma of the bladder without neoadjuvant or adjuvant therapy: long-term results in 1100 patients. Eur Urol 2012;61:1039–47.
[2] Siefker-Radtke AO, Apolo AB, Bivalacqua TJ, Spiess PE, Black PC. Immunotherapy with checkpoint blockade in the treatment of urothelial carcinoma. J Urol 2018;199:1129–42.
[3] Kluth LA, Black PC, Bochner BH, Catto J, Lerner SP, Stenzl A, et al. Prognostic and prediction tools in bladder cancer: a comprehensive review of the literature. Eur Urol 2015;68:238–53.
[4] Schulz GB, Grimm T, Buchner A, Jokisch F, Grabbert M, Schneevoigt BS, et al. Prognostic value of the preoperative platelet-to-leukocyte ratio for oncologic outcomes in patients undergoing radical cystectomy for bladder cancer. Clin Genitourin Cancer 2017;15:e915–21.
[5] Andrlova H, Mastroianni J, Madl J, Kern JS, Melchinger W, Dierbach H, et al. Biglycan expression in the melanoma microenvironment promotes invasiveness via increased tissue stiffness inducing integrin-beta1 expression. Oncotarget 2017;8:42901–16.
[6] Aprile G, Avellini C, Reni M, Mazzer M, Foltran L, Rossi D, et al. Biglycan expression and clinical outcome in patients with pancreatic adenocarcinoma. Tumour Biol 2013;34:131–7.
[7] Gu X, Ma Y, Xiao J, Zheng H, Song C, Gong Y, et al. Up-regulated biglycan expression correlates with the malignancy in human colorectal cancers. Clin Exp Med 2012;12:195–9.
[8] Hu L, Duan YT, Li JF, Su LP, Yan M, Zhu ZG, et al. Biglycan enhances gastric cancer invasion by activating FAK signaling pathway. Oncotarget 2014;5:1885–96.
[9] Jacobsen F, Kraft J, Schroeder C, Hube-Magg C, Kluth M, Lang DS, et al. Up-regulation of biglycan is associated with poor prognosis and PTEN deletion in patients with prostate cancer. Neoplasia 2017;19:707–15.
[10] Liu Y, Li W, Li X, Tai Y, Lu Q, Yang N, et al. Expression and significance of biglycan in endometrial cancer. Arch Gynecol Obstet 2014;289:649–55.
[11] Qian Z, Zhang G, Song G, Shi J, Gong L, Mou Y, et al. Integrated analysis of genes associated with poor prognosis of patients with colorectal cancer liver metastasis. Oncotarget 2017;8:25500–12.
[12] Zaidi AH, Gopalakrishnan V, Kasi PM, Zeng X, Malhotra U, Balasubramanian J, et al. Evaluation of a 4-protein serum biomarker panel-biglycan, annexin-A6, myeloperoxidase, and protein S100-A9 (B-AMP)-for the detection of esophageal adenocarcinoma. Cancer 2014;120:3902–13.
[13] Appunni S, Anand V, Khandelwal M, Seth A, Mathur S, Sharma A. Altered expression of small leucine-rich proteoglycans (Decorin, Biglycan and Lumican): Plausible diagnostic marker in urothelial carcinoma of bladder. Tumour Biol 2017;39:1010428317699112.
[14] Niedworok C, Rock K, Kretschmer I, Freudenberger T, Nagy N, Szarvas T, et al. Inhibitory role of the small leucine-rich proteoglycan biglycan in bladder cancer. PLoS One 2013;8:e80084.
[15] Schaefer L, Tredup C, Gubbiotti MA, Iozzo RV. Proteoglycan neofunctions: regulation of inflammation and autophagy in cancer biology. FEBS J 2017;284:10–26.
[16] Mathias RA, Chen YS, Wang B, Ji H, Kapp EA, Moritz RL, et al. Extracellular remodelling during oncogenic Ras-induced epithelial-mesenchymal transition facilitates MDCK cell migration. J Proteome Res 2010;9:1007–19.
[17] Thakur AK, Nigri J, Lac S, Leca J, Bressy C, Berthezene P, et al. TAp73 loss favors Smad-independent TGF-beta signaling that drives EMT in pancreatic ductal adenocarcinoma. Cell Death Differ 2016;23:1358–70.
[18] Baumgart E, Cohen MS, Silva Neto B, Jacobs MA, Wotkowicz C, Rieger-Christ KM, et al. Identification and prognostic significance of an epithelial-mesenchymal transition expression profile in human bladder tumors. Clin Cancer Res 2007;13:1685–94.
[19] Breyer J, Gierth M, Shalekenov S, Aziz A, Schafer J, Burger M, et al. Epithelial-mesenchymal transformation markers E-cadherin and survivin predict progression of stage pTa urothelial bladder carcinoma. World J Urol 2016;34:709–16.
[20] Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. Nat Rev Clin Oncol 2018;15:81–94.
[21] Robertson AG, Kim J, Al-Ahmadie H, Bellmunt J, Guo G, Cherniack AD, et al. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell 2017;171:540−56.e25.
[22] Lerner SP, McConkey DJ, Hoadley KA, Chan KS, Kim WY, Radvanyi F, et al. Bladder cancer molecular taxonomy: summary from a consensus meeting. Bladder Cancer 2016;2:37–47.
[23] Seiler R, Ashab HAD, Erho N, van Rhijn BWG, Winters B, Douglas J, et al. Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. Eur Urol 2017;72:544–54.
[24] Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507:315–22.
[25] Liberzon A, Subramanian A, Pinchback R, Thorvaldsdottir H, Tamayo P, Mesirov JP. Molecular signatures database (MSigDB) 3.0. Bioinformatics 2011;27:1739–40.
[26] Mari A, Kimura S, Foerster B, Abufaraj M, D'Andrea D, Gust KM, et al. A systematic review and meta-analysis of lymphovascular invasion in patients treated with radical cystectomy for bladder cancer. Urol Oncol 2018;36:293–305.
[27] Fajkovic H, Halpern JA, Cha EK, Bahadori A, Chromecki TF, Karakiewicz PI, et al. Impact of gender on bladder cancer incidence, staging, and prognosis. World J Urol 2011;29:457–63.
[28] Messer JC, Shariat SF, Dinney CP, Novara G, Fradet Y, Kassouf W, et al. Female gender is associated with a worse survival after radical cystectomy for urothelial carcinoma of the bladder: a competing risk analysis. Urology 2014;83:863–7.
[29] Lindgren D, Liedberg F, Andersson A, Chebil G, Gudjonsson S, Borg A, et al. Molecular characterization of early-stage bladder carcinomas by expression profiles, FGFR3 mutation status, and loss of 9q. Oncogene 2006;25:2685–96.

[30] Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. PNAS 2010;107:15449–54.

[31] Xing X, Gu X, Ma T. Knockdown of biglycan expression by RNA interference inhibits the proliferation and invasion of, and induces apoptosis in, the HCT116 colon cancer cell line. Mol Med Rep 2015;12:7538–44.

[32] Hu L, Zang MD, Wang HX, Li JF, Su LP, Yan M, et al. Biglycan stimulates VEGF expression in endothelial cells by activating the TLR signaling pathway. Mol Oncol 2016;10:1473–84.

[33] Xing X, Gu X, Ma T, Ye H. Biglycan up-regulated vascular endothelial growth factor (VEGF) expression and promoted angiogenesis in colon cancer. Tumour Biol 2015;36:1773–80.

[34] Jager T, Becker M, Eisenhardt A, Tilki D, Totsch M, Schmid KW, et al. The prognostic value of cadherin switch in bladder cancer. Oncol Rep 2010;23:1125–32.

[35] Liu B, Miyake H, Nishikawa M, Fujisawa M. Expression profile of epithelial-mesenchymal transition markers in non-muscle-invasive urothelial carcinoma of the bladder: correlation with intravesical recurrence following transurethral resection. Urol Oncol 2015;33:110.e11–18.

[36] Schulte J, Weidig M, Balzer P, Richter P, Franz M, Junker K, et al. Expression of the E-cadherin repressors Snail, Slug and Zeb1 in urothelial carcinoma of the urinary bladder: relation to stromal fibroblast activation and invasive behaviour of carcinoma cells. Histochem Cell Biol 2012;138:847–60.

[37] Roth B, Jayaratna I, Sundi D, Cheng T, Melquist J, Choi W, et al. Employing an orthotopic model to study the role of epithelial-mesenchymal transition in bladder cancer metastasis. Oncotarget 2016. https://doi.org/10.18632/oncotarget.11009.

[38] Choi W, Czerniak B, Ochoa A, Su X, Siefker-Radtke A, Dinney C, et al. Intrinsic basal and luminal subtypes of muscle-invasive bladder cancer. Nat Rev Urol 2014;11:400–10.